Drug Design

Aminoglycoside Microarrays To Study Antibiotic Resistance**

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Aminoglycosides are broad-spectrum antibiotics against a variety of clinically important bacteria. Their antibacterial effect is due to binding to bacterial 30S ribosomes and inhibiting protein synthesis. The therapeutic efficacy of aminoglycosides, however, has decreased recently because of increased antibiotic resistance.[1,2] Bacteria use several mechanisms to achieve resistance including decreased uptake of the drug into cells, mutation of the target, binding of the drug to proteins, and covalent modification of the drug by enzymes.[1,2] Enzymatic modification is the most common mechanism leading to aminoglycoside resistance. The result of aminoglycoside modification is a large decrease in binding

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affinity to the therapeutic target.[3] The development of high-throughput methods to identify compounds that weakly bind to resistance-causing proteins and strongly bind to therapeutic targets would facilitate the discovery of improved antibiotics. Such methods also allow for a better understanding of the interactions of antibiotics with therapeutic targets and resistance-causing proteins. Microarrays,[4–8] created by the immobilization of small molecules onto glass surfaces, provide a versatile platform for rapidly screening several thousand potential antibiotics in parallel for binding to therapeutic targets and resistance-causing proteins. These screens are particularly attractive since only miniscule amounts of potential antibiotics, therapeutic targets, and resistance-causing proteins are needed, and thus the limitations of current screening methods can be overcome.

Here we report the construction of aminoglycoside microarrays and their use in probing the binding of aminoglycosides to resistance-causing proteins. Two aminoglycoside acetyltransferases that cause antibiotic resistance, 2′-acetyltransferase (AAC(2′)) from *Mycobacterium tuberculosis*[9] and 6′-acetyltransferase (AAC(6′)) from *Salmonella enterica*[10] were used as examples. Hybridization of these enzymes to aminoglycoside arrays show that each immobilized aminoglycoside interacts with both AAC(2′) and AAC(6′). Furthermore, delivery of as little as picomoles of aminoglycoside to slides was sufficient for binding to be observed (Figure 1). The signal from mannose, a negative control, was much lower, indicating that specific interactions were detected.

Binding of aminoglycoside antibiotics to resistance enzymes can be grouped into two categories based on the fluorescence signal from the highest concentration spot (Figure 1). For AAC(6′), the strongest fluorescence arose from binding to amikacin, tobramycin, kanamycin B, lividomycin, neomycin, and ribostamycin. Kanamycin A, apramycin, paromomycin, gentamycin, and neamine resulted in lower signals. For AAC(2′), the strongest signals were observed for amikacin, paromomycin, tobramycin, and ribostamycin while kanamycin B, apramycin, kanamycin A, neamine, neomycin, lividomycin, and gentamycin displayed weaker signals.

To determine how hybridization of AAC(6′) to the aminoglycoside arrays correlates with protein–aminoglycoside binding measurements in solution, we made comparisons to a calorimetric study of aminoglycoside binding to AAC(6′).[11] This analysis shows a strong correlation between these different types of measurements. Calorimetry showed that ribostamycin, tobramycin, lividomycin, and neomycin have the strongest affinities, whereas kanamycin B, paromomycin, gentamycin C, kanamycin A, and amikacin had lower affinities to AAC(6′). Generally the array data and the calorimetry data correlate well when the affinities are classified as strong and stronger with the exception of amikacin, which had one of the strongest fluorescence signals with both enzymes. Amikacin, unlike most of the other aminoglycosides, contains two primary amino groups; the sterically least encumbered amino group is six carbon atoms removed from the 2,4-deoxystreptamine core. Thus, amikacin may be immobilized onto the surface differently than the other aminoglycosides, giving rise to the more intense signal.

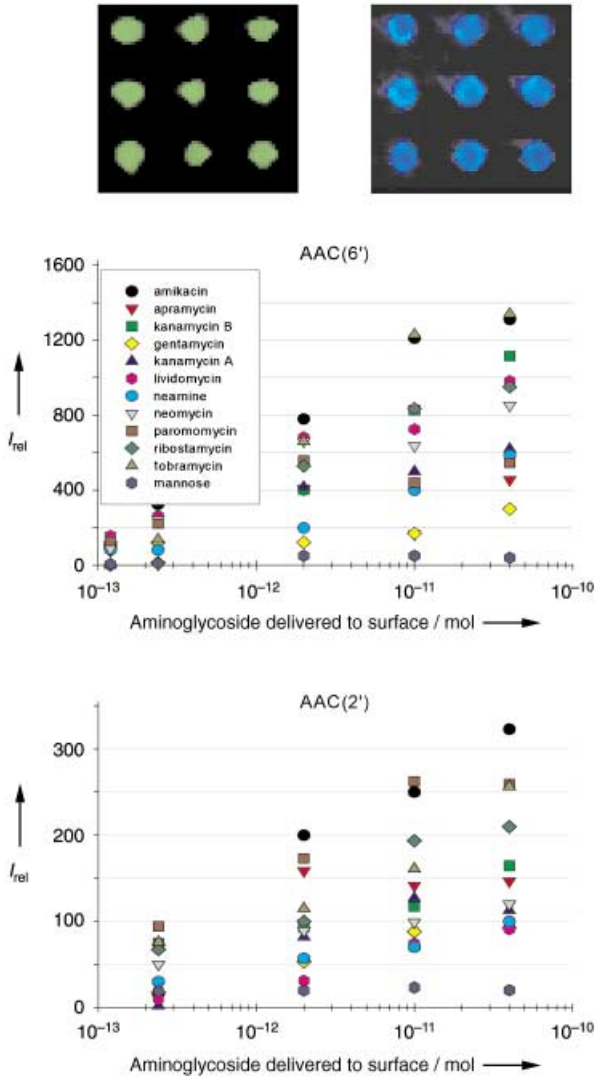
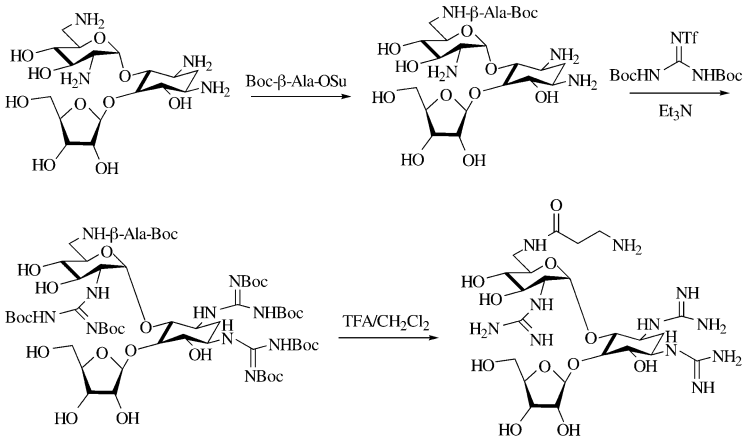


Figure 1. Top: Aminoglycoside microarray after hybridization with AAC(6′) (green) and AAC(2′) (blue). Bottom: Fluorescence intensities for the arrays of antibiotic hybrids after binding to the aminoglycosides.

In an effort to find inhibitors of antibiotic-resistance enzymes, a library of aminoglycoside mimetics was synthesized, arrayed, and screened for tight binding to by AAC(2′) and AAC(6′). Guanidinoglycosides[12,13] are an attractive set of aminoglycoside analogues for several reasons: 1) They are readily synthesized from aminoglycosides. 2) Their increased positive charge may allow them to bind more tightly to the aminoglycoside binding pocket present in resistance-causing enzymes that contain several negatively charged amino acids.[14] 3) The difference in the pK_a values of guanidino (pK_a ~ 12.5) and amino groups (pK_a ~ 8.8) suggests that guanidinoglycosides may not be substrates for AAC(2′) and AAC(6′).

A diverse set of guanidinoglycosides was synthesized (Scheme 1) by reacting each aminoglycoside with Boc-β-Ala-OSu to introduce a primary amino linker for immobilization, which was used to normalize surface loading for each library component. Guanidinylation using *N*,*N*′-di(Boc)-*N*″-triflyl-



Scheme 1. The synthesis of an aminoglycoside mimetic library is illustrated by the synthesis of 6′-*N*-β-alanine-1,3,3′-*N*-guanidinoribostamycin; all other compounds were synthesized similarly. Boc = *t*-butoxycarbonyl, Tf = trifluoromethanesulfonyl, TFA = trifluoroacetic acid.

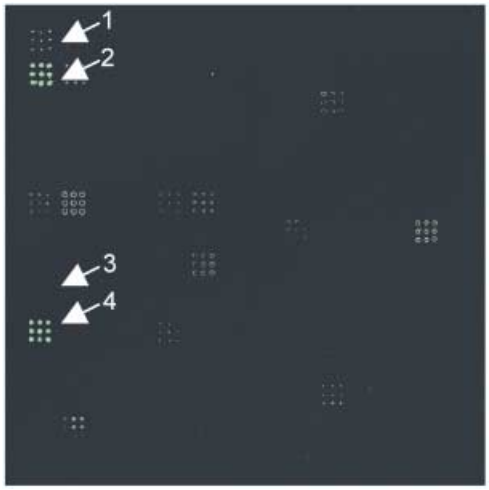


Figure 2. An array containing guanidinoglycosides and aminoglycosides that was incubated with fluorescein-labeled AAC(6′). Arrows point to 3×3 blocks of repetitive spots (spot diameter ≈150 μM) on the array that correspond to β-Ala-neomycin (1), β-Ala-guanidinoneomycin (2), β-Ala-ribosytamycin (3), and β-Ala-guanidinoribostamycin (4).

guanidine[12] typically required at least three days and reactions were stopped when mass spectrometry analysis indicated that no partially guanidinylated compounds were present. Treatment with a solution of trifluoroacetic acid (50 % CH_2Cl_2)[12] afforded the desired guanidinylated products in good overall yield (typical yields per step > 90 %).

For a direct comparison of the effect of guanidinylation, both the guanidinoglycosides and the aminoglycosides equipped with a β-alanine linker were arrayed onto glass slides. In all cases after incubation of the arrays with fluorescently labeled AAC(2′) and AAC(6′), the arrayed guanidinoglycosides exhibited a stronger signal for binding to AAC(2′) and AAC(6′) than the corresponding aminoglycosides. The largest enhancement, sevenfold for AAC(2′) and eightfold for AAC(6′), was observed with β-Ala-guanidinoribostamycin (Table 1 and Figure 2). Based on these studies and calorimetry experiments that demonstrate that ribostamycin has the highest affinity to for AAC(6′), we chose β-Ala-guanidinoribostamycin for more detailed studies.

To determine if β-Ala-guanidinoribostamycin is a substrate for AAC(2′) or AAC(6′), kinetics experiments were performed at 37 °C and monitored spectrophotometically.[10] While ribostamycin is completely consumed after 10 min, β-Ala-guanidinoribostamycin did not react with either AAC(2′) or AAC(6′) (data not shown). In addition, β-Ala-guanidinoribostamycin inhibits both AAC(2′) and AAC(6′)(Table 2). In each case, 6′-β-Ala-guanidinoribostamycin was a noncompetitive inhibitor of the binding of ribostamycin to both AAC(2′) and AAC(6′). Data from the kinetics experiments was used to determine the effect on both K_{ii}, the intercept inhibition constant, and K_{is}, the slope inhibition constant. We found that 6′-β-Ala-guanidinoribostamycin inhibits AAC(6′) and AAC(2′) with K_{ii} and K_{is} values in the 10–200 micromolar range. The observation that 6′-β-Ala-guanidinoribostamycin is a noncompetitive inhibitor suggests that it binds to both the free enzyme and the acetyl-CoA enzyme complex, which is also suggested to be how lividomycin noncompetitively inhibits AAC(6′).[10]

In conclusion, we have developed a microarray method for screening antibiotics for binding to resistance-causing enzymes. When this method is used in combination with screening for tight binding of antibiotics to therapeutic

Table 1: Enhancement in binding to AAC(2′) and AAC(6′) by addition of guanidino groups to selected aminoglycosides.[a]

Aminoglycoside	AAC(6′)	AAC(2′)
kanamycin A	2.1	1.1
kanamycin B	1.6	5.5
neomycin	3.2	2.5
ribostamycin	7.5	6.7
paromomycin	1.4	1.1
lividomycin	1.1	1.8

[a] The enhancement is the increase in signal due to the presence of the guanidino groups and is calculated by dividing the signal from the guanidinoglycoside by the signal from the corresponding aminoglycoside.

Table 2: Inhibition of AAC(2′) and AAC(6′) by 6′-*N*-β-alanine-1,3,3′-*N*-guanidinoribostamycin.[a]

Aminoglycoside	K_M [μM]	V_{max} [10^{-2} min^{-1}][b]	K_{ii} [μM]	K_{is} [μM]
		AAC(6′)		
ribostamycin	4.4(±0.8)	18(±0.1)	92(±20)	17(±8)
kanamycin A	12(±3)	2.8(±0.2)	23(±7)	31(±15)
		AAC(2′)		
ribostamycin	4.4(±0.7)	4.4(±1.8)	134(±40)	39(±16)
kanamycin B	0.7(±0.2)	2.6(±0.2)	61(±20)	200(±50)

[a] Experiments to determine inhibition constants were performed with variable concentrations of aminoglycoside and inhibitor. For details see the Supplementary Information. [b] Rates are $\Delta A_{412}\,min^{-1}$.

targets, new antibiotics that are weak binders to resistance-causing enzymes and tight binders to therapeutic targets can be identified. This new method was used to screen a series of aminoglycoside mimetics for high-affinity binding to the resistance-causing enzymes AAC(2′) and AAC(6′) in an effort to discover inhibitors of resistance. These screens identified 6′-β-Ala-guanidinoribostamycin as a high-affinity binder to both AAC(2′) and AAC(6′). Further experiments show that 6′-β-Ala-guanidinoribostamycin is not a substrate for either resistance enzymes and is an inhibitor of acylation of several clinically important antibiotics. This approach should prove valuable for screening libraries of compounds to discover improved antibiotics that evade current modes of bacterial resistance.

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